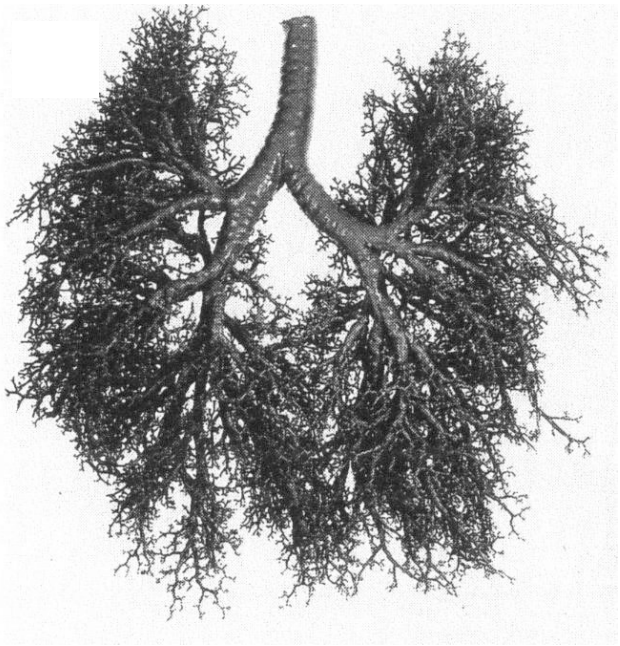
LUNG BIOMARKERS FOR TOXICOLOGY STUDIES



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"BIOMARKERS" DISCUSSED

Respiratory function
Lung imaging
Bronchoalveolar lavage
Tissue chemistry
Special stains & immunohistochemistry
Cell proliferation
Morphometrics
Gene expression

} Definition
Uses
Advantages
Disadvantages

Suitability for complementing NTP hazard assessment bioassays

- Useful for detecting and characterizing adverse responses
- Acceptably standardized methods
- Generally accepted interpretation of results & extension to humans
- Enhances evaluation of hazard by current definitions

RESPIRATORY FUNCTION

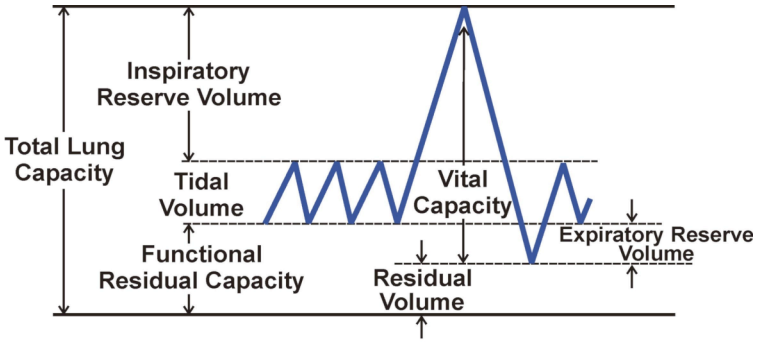
Ventilation

Respiratory frequency = f

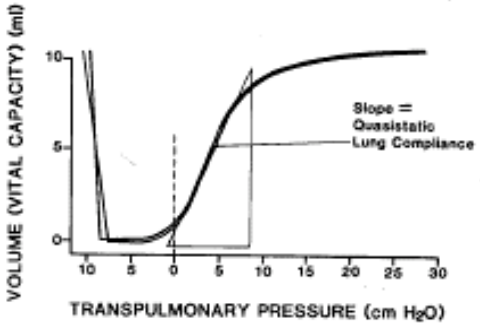
Tidal volume = V_T

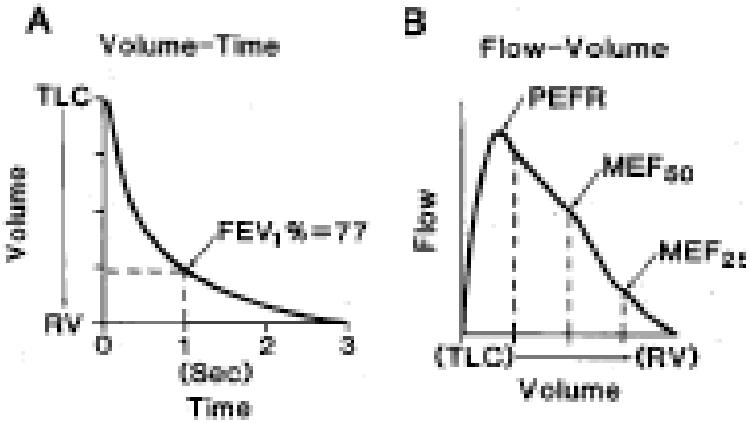
Minute volume = V_E

Volumes

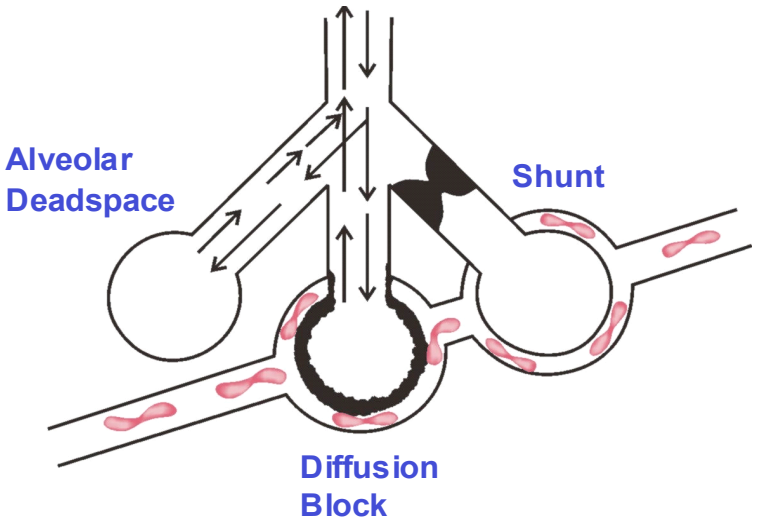


Mechanics





Diffusion and Perfusion

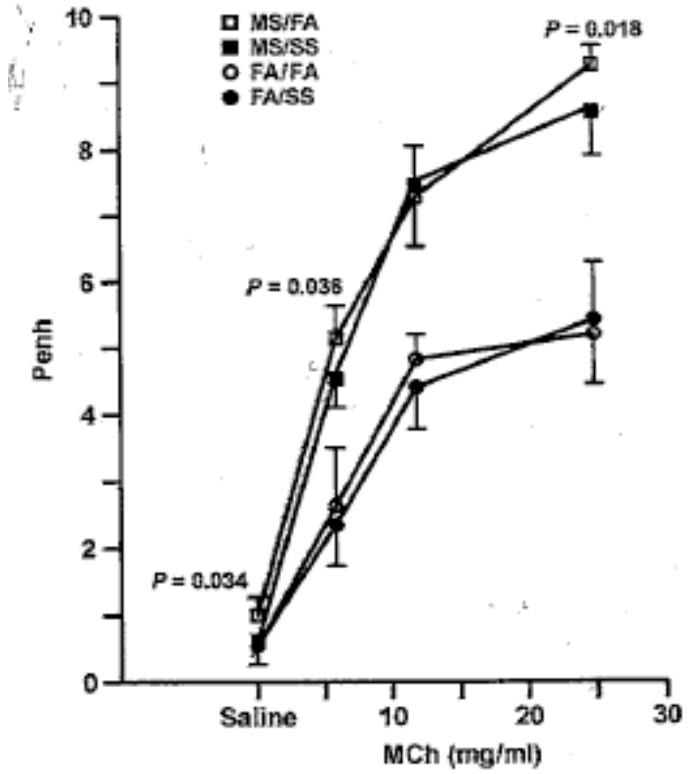


(CO Diffusing Capacity)

Mauderly, Chapt. 13 in: *Concepts in Inhalation Toxicology*, McClellan & Henderson, Eds., Taylor & Francis, 1995

RESPIRATORY FUNCTION

Airway responsiveness



Exposed *In Utero* to mainstream smoke

No *In Utero* Exposure

Singh et al., *Am. J. Resp. Crit. Care Med.* 168: 342, 2003

RESPIRATORY FUNCTION

Uses

- Describes functional manifestation of structural changes
- Places functional impacts of disorders into clinical context
- Provides correlates to humans

Advantages

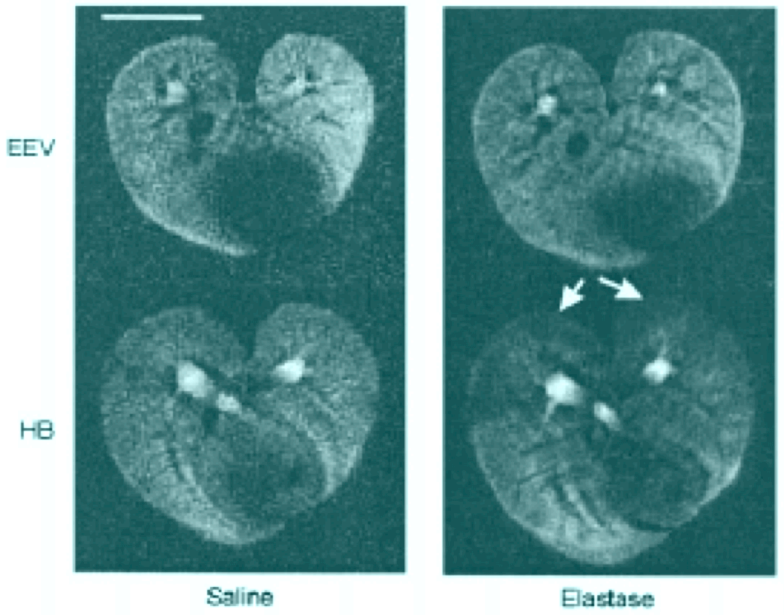
- Can be done non-destructively (with anesthesia)
- Established methods and interpretation

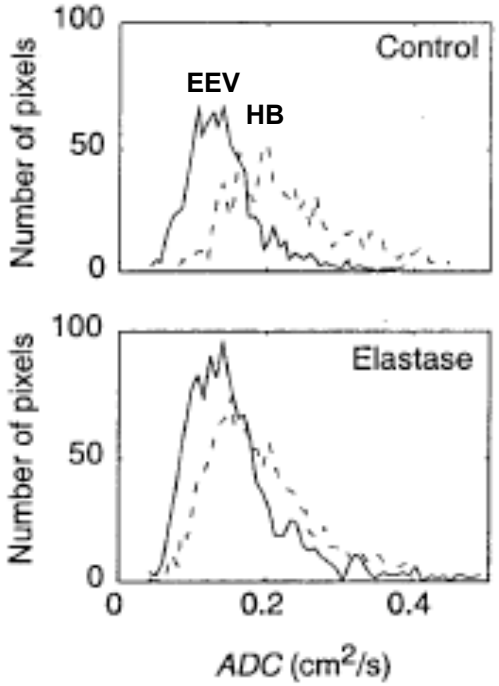
Disadvantages

- Most tests require specialized equipment and expertise
- Not very sensitive to subtle or scattered tissue changes
- Not a substitute for histopathology or other indicators
- Does not determine type of morphological abnormality

LUNG IMAGING

Gas density images of rat lungs using MRI measurement of apparent gas diffusion coefficient of hyperpolarized He





Diffusion coefficients of middle third of lungs

Chen et al, Proc. Nat. Acad. Sci. 97: 11478, 2000

Image lungs using x-ray, MRI, PET scan, etc.

With or without contrast media to highlight structures

LUNG IMAGING

Uses

- Detection and staging size and distribution of lesions
- Following abnormalities with time
- Provides correlates to humans

Advantages

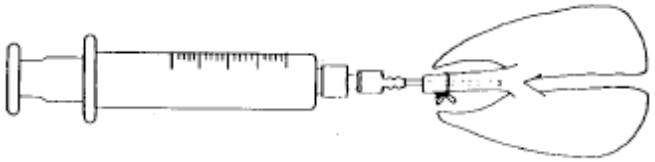
- Non-destructive (requires anesthesia)

Disadvantages

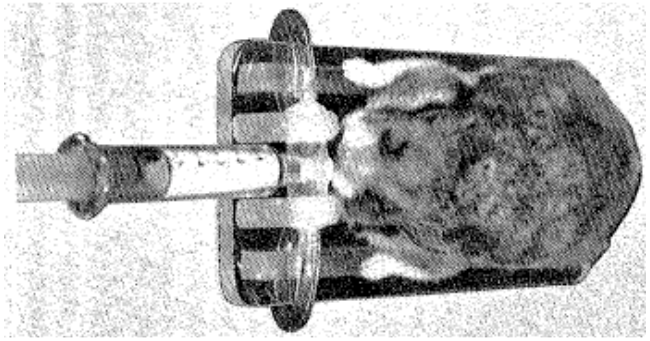
- Marginal resolution of some methods for imaging rodents (e.g., x-ray)
- Some methods require sophisticated/expensive equipment (e.g., MRI)
- Not very sensitive to subtle or scattered, focal tissue changes

BRONCHOALVEOLAR LAVAGE

Biomarkers in fluid instilled into and withdrawn from airways



Henderson, Exper. Toxicol. Pathol. 57: 155, 2005



Mauderly, *Lab An. Sci.* 27:255, 1977

Cells (RBC, WBC, differentials, collections for cell morphology, DNA, or function)

Protein (total, albumin, hemoglobin, proteomics)

Enzymes (Lactate dehydrogenase, β-blucuronidase, alkaline phosphatase)

Cytokines/chemokines (interleukins, TNFα. MIP-2, etc.)

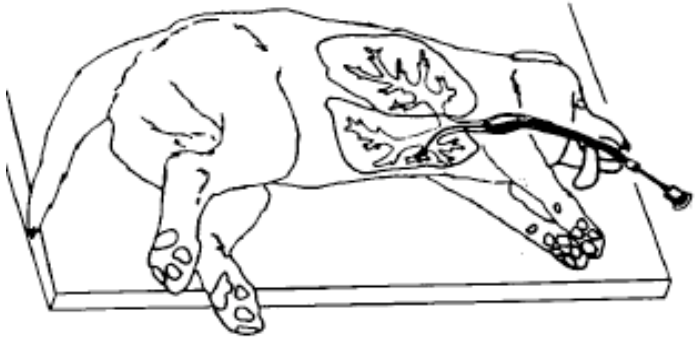
Growth factors

Glutathione (total, reduced)

Fibronectin

Antibodies

Elastin/collagen breakdown products



BRONCHOALVEOLAR LAVAGE

Uses

- Detection of inflammation, cytotoxicity, oxidative stress, lung tissue metabolism, allergic reactions
- Provides correlates to humans

Advantages

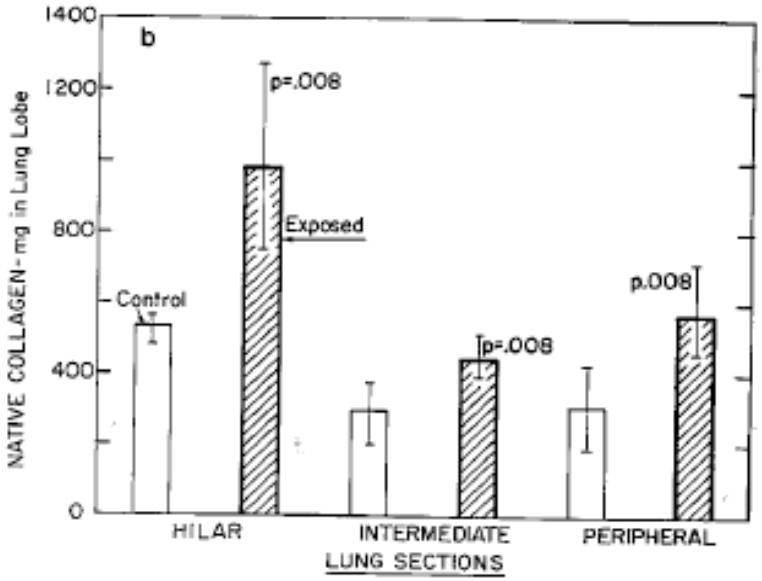
- Readily done at necropsy (can be done on one lobe)
- Assays are straightforward (kits available for many variables)
- Established methods and interpretation
- Non-destructive to tissue (can be coupled with other assays)

Disadvantages

- Reflects responses in airspaces, not necessarily in tissues
- Modest ability to detect or stage chronic lung disease (e.g., fibrosis, emphysema, cancer)
- All species can be lavaged in vivo, but not all survive or completely clear atelectatic spots (mice, S. hamsters, rabbits, NHP, dogs do – rats, guinea pigs, & gerbils do not)

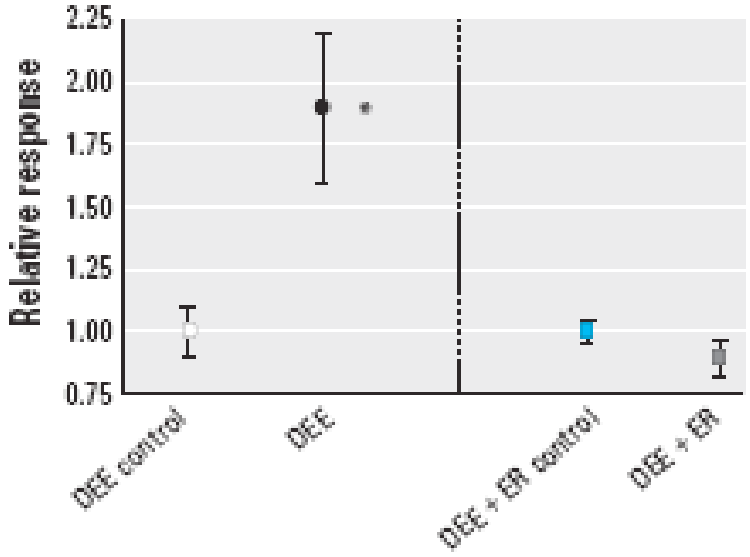
TISSUE CHEMISTRY

Lung Collagen
Radiation Pneumonitis-Fibrosis



Pickrell et al., *Rad. Res*. 74:363, 1978

HO-1 in Lung
With and Without Diesel Emissions Control



McDonald et al., *EHP*, 112: 1307, 2004

Chemical analysis to determine content of target material

Structural (e.g., collagen) or functional (e.g., hemoxygenase-1, glutathione) analytes

TISSUE CHEMISTRY

Uses

- Measure changes in concentration of specific structural components
- Measure functional status

Advantages

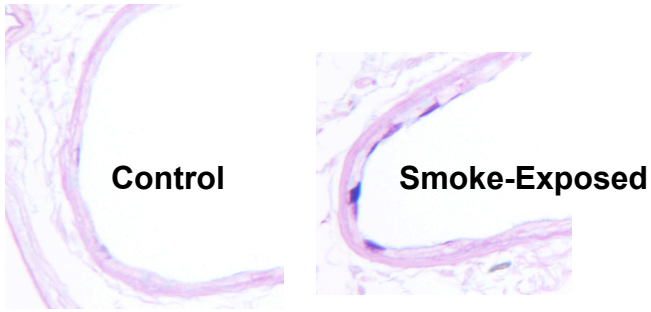
- Provides quantitative information

Disadvantages

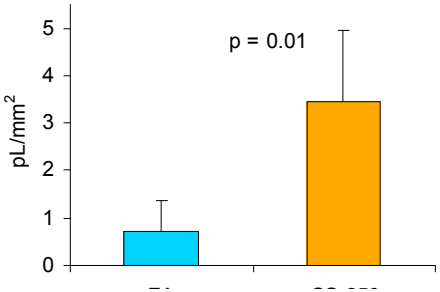
- Isolation to particular anatomic site is dependent on level of tissue dissection (whole lung, lobe, microdissection)
- Averages all structures and cells within sample
- Destructive

SPECIAL STAINS AND IMMUNOHISTOCHEMISTRY

Alcian Blue/Periodic Acid Schiff Stain



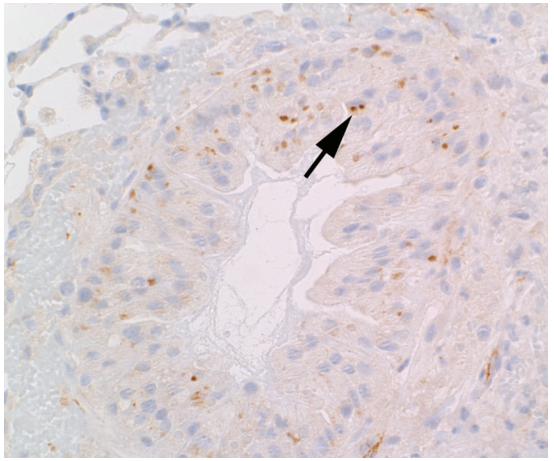
Mucosubstance Volume in Airways of Mice Exposed to Cigarette Smoke



March *et al.*, *Toxicol. Sci.* 92:545, 2006

Phosphorylated IKKα Stain

(inhibitor of nuclear factor Kβ kinase)



Activation of NFKβ in airway of mouse infected with cowpox virus

J. Hutt, LRRI, personal communication, 9/15/06

Stain bound chemically (collagen, elastin, mucus, etc.)

Stain linked to antibody (BrdU, Bcl-2, cytokeratin, NFKβ, etc.)

SPECIAL STAINS AND IMMUNOHISTOCHEMISTRY

Uses

- Enhance identification, visualization, and quantitation of structural/chemical abnormalities

Advantages

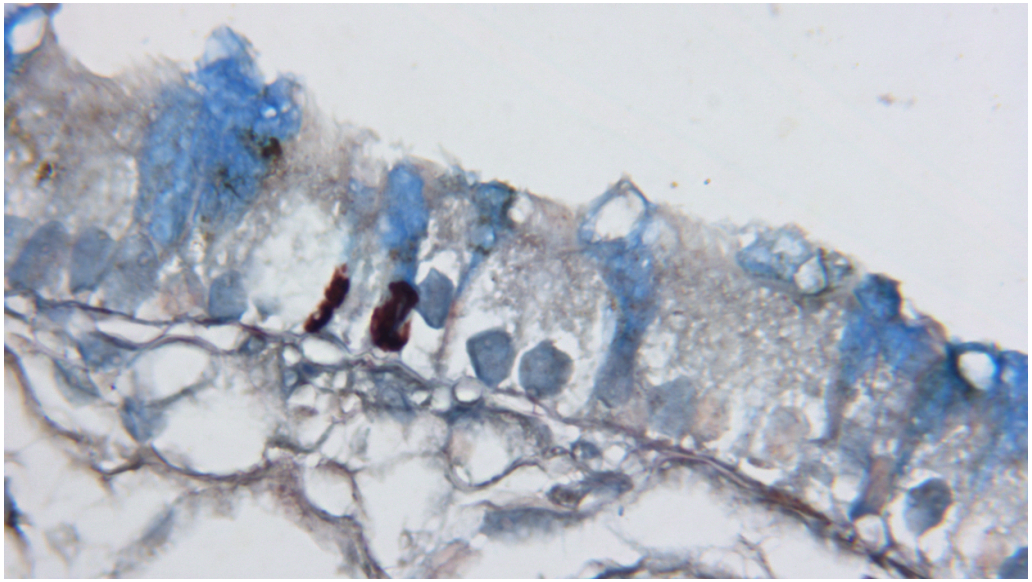
- Provides information on anatomic location and variation
- Can target very specific materials
- Can be quantitative

Disadvantages

- Reliant on sampling/sectioning scheme
- Provides 2-dimensional view
- Immunohistochemistry depends on availability of antibody

CELL PROLIFERATION

BrdU staining (brown) of airway epithelial cells



Y. Tesfaigzi, LRRI, personal communication, 9/15/06

Identify dividing cells using nuclear marker

Can label ante-mortem (e.g., BrdU - thymidine analog) or post-mortem (e.g., Ki67 - nuclear antigen)

Often measured as cells/mm basal lamina

CELL PROLIFERATION

Uses

- Assess cell turnover rates
- Detect and measure proliferative events

Advantages

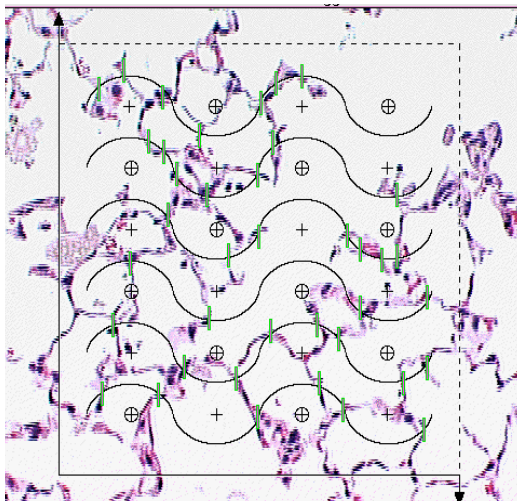
- Provides quantitative information on proliferative status
- Detect “harbinger” cellular responses

Disadvantages

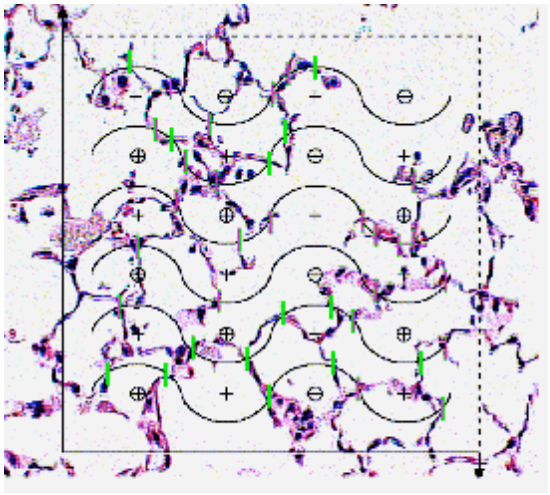
- Ante-mortem labeling by injection provides limited time window
- Prolonged ante-mortem labeling requires repeated injections or implantation of osmotic pumps
- Concurrent markers often necessary to confirm cell type
- Manual counting takes time
- Proliferation rate may be only slightly above background in chronic conditions (may miss time course of early events)

MORPHOMETRICS

Horizontal Cycloid Grid Overlying Alveoli



Intercepts with tissue



Width of alveoli

Air space enlargement

L_m = length ÷ ΣI_{spt}

$V_{Vair} = \Sigma P_{air} \div \Sigma P_L$

Tissue destruction

$V_{Vspt} = \Sigma P_{spt} \div \Sigma P_L$

$S_a = (4 \times V_L) \div L_m$

T. March, LRRI, personal communication, 9/15/06

Quantitative analysis of structural dimensions

1) Extent and distribution of abnormalities (e.g., collagen)

2) Morphological dimensions (e.g., airspaces, airway and vessel walls)

Stereology (extrapolation to 2-dimenional surface density or 3-dimensional volume density by 2-dimensional point counting)

Linear frequency rates (counts of events per unit distance, e.g., cells per mm basal lamina)

MORPHOMETRICS

Uses

- Provides quantitative assessment of structural changes

Advantages

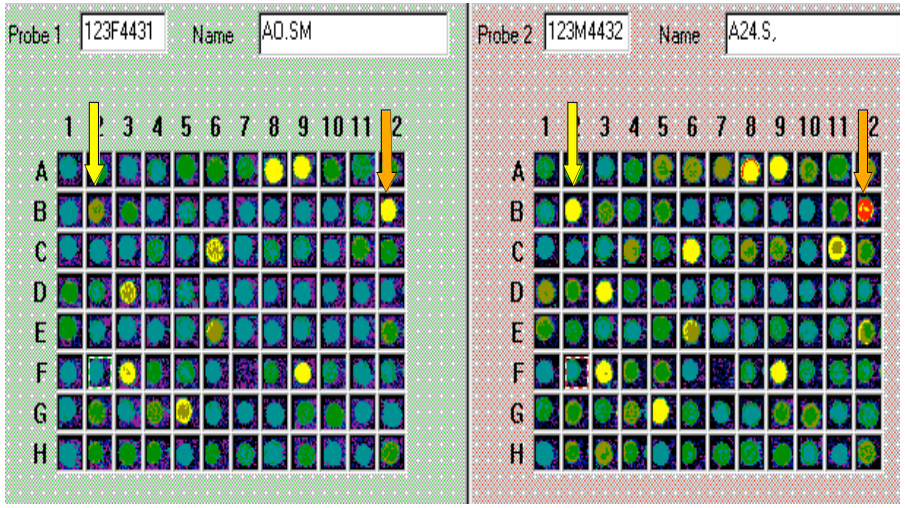
- Adjunct to subjective, non-quantitative assessment/scoring
- Supports statistical analyses

Disadvantages

- Can be tedious
- Accuracy highly dependent on:
  1) identification of structures
  2) sampling and computational schemes
     - number & location of sections
     - number of events counted
     - accuracy of normalization variable (e.g., lung volume)

GENE EXPRESSION

Control **Diesel-exposed**



Stringency	Significant Differences	False Positives
P<0.05	513	183 (36%)
P<0.01	198	36 (18%)
FDR = 0.10	99	10 (10%)
FDR <1 gene	32	<1 (<3%)

(3649 reads of 4273)

Liekauf, presented at NERC Annual Meeting, May 2003, unpublished

Expression of genes, using mRNA isolated from lung

rtPCR/gel or real-time rtPCR for specific genes

Micro-array survey (standard or custom arrays)

Comparison with control expression level

GENE EXPRESSION

Uses

- Assess activation status of specific gene(s)
- Screen for detecting and categorizing exposure effects
- Generate hypotheses about mechanisms of response

Advantages

- Sensitive – likely to detect exposure-related differences
- Patterns may be descriptive of response type and mechanisms

Disadvantages

- Usually requires fresh or frozen tissue
- Integrates across all cell types in sample
- Gives information for one point in time
- Gene expression may not reflect protein production
- Expression of “clock” genes is affected by sample time
- Micro-arrays incur data analysis challenges
- Utility dependent on understanding links between gene expression and outcomes relevant to human hazard (limited number of well-established extrapolation pathways)

PROTEOMICS

Uses, Advantages, Disadvantages

- Generally same as for gene expression
- Advantage: measures actual product
- Advantage or disadvantage: measures total product present, regardless of when produced

SUMMARY

- All of these biomarkers (and others) could be useful
- Probably none should be included routinely in all bioassays
- These are not substitutes for conventional bioassay endpoints
- Most of these are not high-throughput approaches
- Selection depends on:
    1) Outcome of concern
    2) Nature of evidence for human hazard
    3) Current regulatory definitions of “hazard” and “adverse effect”
    4) Extent to which mechanisms and time course are of interest